Metabolic Disposition of Ivermectin in Swine

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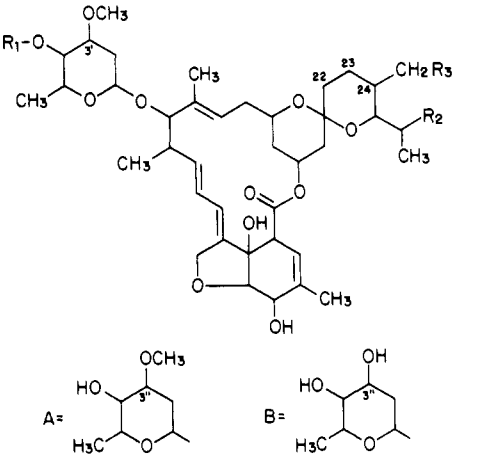
Tissue residue distribution and metabolism of tritium-labeled ivermectin have been studied in swine dosed subcutaneously at 0.4 mg/kg of body weight. The residue distribution pattern among edible tissues (liver, kidney, muscle, and fat) was similar to those found in cattle, sheep, and rats, with highest levels in fat and liver tissues ranging between 78 and 654 ppb within 7 days after dosing. The parent drug was the major radioactive residue in liver and fat, accounting for >50% of total radioactivity up to 7 days and ~30% after 14 days. The major liver metabolites were identified as 3″-*O*-desmethyl-H_2B_{1a} and 3″-*O*-desmethyl-H_2B_{1b} by chemical derivatization and mixed-sample HPLC cochromatography with in vitro metabolites from swine liver microsomal incubations. As in other species studied, good correlation has been observed between in vitro and in vivo metabolism. The drug was essentially eliminated by fecal and biliary excretion.

Ivermectin is one of the most potent antiparasitic agents developed in recent years (Chabala et al., 1980). The compound has become one of the most widely used anthelmintic agents for food-producing animals as well as for other species such as horses and dogs. Ivermectin is the 22,23-dihydro derivative of avermectin B_1, a macrocyclic lactone produced by an actinomycetes, *Streptomyces avermitilis* (Burg et al., 1979; Egerton et al., 1979; Miller et al., 1979). Ivermectin is marketed as a mixture of two compounds, i.e., dihydroavermectin B_{1a} (H_2B_{1a}, I) and dihydroavermectin B_{1b} (H_2B_{1b}, II). Their structures are shown in Figure 1. The composition of these two compounds in ivermectin is $H_2B_{1a} \geq 80\%$ and $H_2B_{1b} \leq 20\%$. The two compounds differ only by one methylene group at the C_{26} position.

We have previously reported metabolic disposition studies of ivermectin in cattle, sheep, and rat (Chiu et al., 1986; Campbell et al., 1983) as well as the in vitro metabolism of ivermectin by hepatic microsomes prepared from swine (Chiu et al., 1984, 1987). To all species studied, ivermectin was administered subcutaneously or orally at 0.3–0.4 mg/kg of body weight. We have concluded on the basis of these studies that the metabolic disposition of radiolabeled ivermectin was similar in cattle, sheep, and rat while differences were noted in swine. The parent drug was the major liver residue component in all species studied. The major liver metabolite in cattle, sheep, and rat was 24-(hydroxymethyl)-H_2B_{1a} (V); those in swine were 3″-*O*-desmethyl-H_2B_{1a} (IX) and 3″-*O*-desmethyl-H_2B_{1b} (X). In this paper, we report results of studies in swine with tissue residue distribution, as well as isolation and identification of major in vivo metabolites from the liver.



		R_1	R_2	R_3
I	H_2B_{1a}	A	CH_2CH_3	H
II	H_2B_{1b}	A	CH_3	H
III	H_2B_{1a}-Monosaccharide	H	CH_2CH_3	H
IV	H_2B_{1b}-Monosaccharide	H	CH_3	H
V	24-OH-H_2B_{1a}	A	CH_2CH_3	OH
VI	24-OH-H_2B_{1b}	A	CH_3	OH
VII	24-OH-H_2B_{1a}-Monosaccharide	H	CH_2CH_3	OH
VIII	24-OH-H_2B_{1b}-Monosaccharide	H	CH_3	OH
IX	3″-O-Desmethyl-H_2B_{1a}	B	CH_2CH_3	H
X	3″-O-Desmethyl-H_2B_{1b}	B	CH_3	H

Figure 1. Structures of ivermectin and metabolites.

MATERIALS AND METHODS

Chemicals. All organic solvents were of HPLC grade from Fisher Scientific (West Haven, CT) or MCB Manufacturing Chemists (Cincinnati, OH). Water was doubly distilled and filtered with 0.45-μm filters (Millipore, Bedford, MA). Unlabeled H_2B_{1a} and H_2B_{1b} were obtained from the Chemical Data Department, MSDRL. Tritium-labeled H_2B_{1a} and H_2B_{1b} were labeled at C_{22}–C_{23} positions by catalytic tritiation of avermectin B_{1a} and B_{1b} as reported previously (Campbell et al., 1983). The two radioactive compounds were of 98.5% radiopurity and at specific activities of ~60 mCi/mg. For dosing animals, the compounds were diluted with unlabeled H_2B_{1a} or H_2B_{1b} separately to specific activities of 0.1 mCi/mg and then mixed at a ratio of H_2B_{1a}:H_2B_{1b} of 80:20. The dosing solution was prepared in formulation A [propyleneglycol/glycerol formal, 60:40 v/v, containing 5% poly(vinylpyrrolidone)] at a concentration of 7.21 mg/g of solvent. For the ivermectin assay, standard solutions of ivermectin were prepared in absolute ethanol at about 1 mg/mL. The concentration was determined from absorbance at 245 nm of 1:50 diluted standard solution and calculated on the basis of the absorption coefficient of ivermectin $A_{cm}^{1\%} = 365$).

Animal Experiments. Fifteen Yorkshire barrows of body weight between 20 and 27 kg were used in the study. They were provided with a typical swine ration and water ad libitum. Twelve barrows assigned to medication were dosed with 400 μg/

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kg of body weight of tritium-labeled ivermectin in formulation A as a single subcutaneous injection in the neck. Three barrows were randomly assigned to each of four withdrawal times (1, 7, 14, and 28 days postdose) when they were slaughtered. Muscle, liver, kidneys, fat, injection site tissue (250 g), and plasma were collected from all barrows at this time. In addition, from one randomly selected barrow in each withdrawal group, the following samples were collected: adrenals, bile, bone marrow, brain, cecum, colon, heart, small intestine, lymph gland, pancreas, spleen, stomach, thymus, thyroid, and tongue. All materials were homogenized in water and frozen in preparation for combustion assay of the total radioactivity. Three unmedicated barrows were used as controls, which supplied control edible tissues and plasma. Daily collection of urine and feces was made from three swine of the 28-day withdrawal group during the first week postdose. Urine and feces were weighed, and feces were homogenized.

Assay of Radioactivity of Tissues and Body Fluids. Tissues or feces samples from each animal at each time point were homogenized in water (1:3 dilution) using a Virtis homogenizer (Virtis, New York). Aliquots (~0.5 g) of the homogenate were removed and combusted with a Packard Model 306 sample oxidizer (Packard, Downers Grove, IL) after drying overnight at room temperature. All samples were analyzed in duplicate and reported as averages. Detection limits were determined by analyses of control tissue samples.

About 125 mg of fat, bile, and bone marrow was combusted directly without homogenization. For injection site samples, due to the fatlike heterogeneous nature of the tissue, neither combustion nor homogenization would be satisfactory. The following procedure was developed for the assay: 2 g of tissue was macerated in a 15-mL centrifuge tube to which 20 mL of toluene was added, and the mixture was heated in an oil bath at 100–110 °C for 1 h. The toluene extract was decanted, and the residue was reextracted with 20 mL of toluene as described. Aliquots of the combined toluene extracts were assayed by scintillation counting in toluene/ethanol (70:30) cocktail (Packard). The residue remaining from the two extractions was dried at 100 °C, and an aliquot was weighed and combusted as described for the tissue samples. Radioactivity in the tritiated water recovered after combustion was measured by scintillation counting in Monophase 40 (Packard). All radioactivity measurements were made in a Packard scintillation spectrometer Model 3355 or 3310 with quench corrections based on the external standard method. Background radioactivity was measured by using the appropriate control fluid, feces, or edible tissues (liver, kidney, muscle, and fat). Liver was used as the control for tissues other than the edible tissues. Recoveries of radioactivity from the sample oxidizer were measured by assay of control samples of each tissue with an added known amount of tritium radioactivity. Recoveries in this study ranged between 97 and 99%. Efficiency of 36–40% was generally obtained with tritium radioactivity.

Extraction of Radioactive Ivermectin Residues from Liver, Kidney, Muscle, and Fat Tissue. Tissue samples (2–10 g) were homogenized in equal volumes of methylene chloride with a Virtis homogenizer for 2 min at high speed. For RIDA, unlabeled ivermectin carrier (500–750 μg) was added to the tissue suspension before homogenization. The homogenate was centrifuged at 2000 rpm to separate the tissue and the extract. The supernatant was withdrawn and evaporated to dryness under reduced pressure with a rotary evaporator (Brinkman, New York). The tissue residue was washed once more with methylene chloride/acetone (1:1), and the washings were combined with the first extract. The dried extract was redissolved in 20 mL of methylene chloride and added to a slurry of silica gel (60–200 mesh, J. T. Baker, Phillipsburg, NJ) prepared in methylene chloride. The slurry was allowed to stand for 2 min before being filtered through a medium-pore sintered glass funnel (150 mL) and washed with 88 mL of the same solvent mixture. The silica gel was washed further with methylene chloride (76 mL) and ethyl acetate (149 mL). Aliquots of these washings were assayed for radioactivity by scintillation counting. The ethyl acetate wash was evaporated to dryness, redissolved in 2 mL of methylene chloride, and passed through a silica gel Sep-Pak cartridge. The cartridge was eluted with two 2-mL aliquots of methylene chloride followed by 8 mL of ethyl acetate. The ethyl acetate eluate was evaporated to dryness and redissolved in 1 mL of methanol before HPLC analysis.

In Vitro Metabolites of Ivermectin. Metabolites of tritium-labeled or unlabeled H_2B_{1a} and H_2B_{1b} were prepared by incubation with swine liver microsomes as published by our laboratories (Chiu et al., 1984). All in vitro metabolites, i.e., 3″-*O*-desmethyl-H_2B_{1a} (3″-DM-H_2B_{1a}, IX) and 3″-*O*-desmethyl-H_2B_{1b} (3″-DM-H_2B_{1b}, X) were purified by repetitive HPLC and structures confirmed by NMR and MS analyses.

Partial Acid Hydrolysis of Ivermectin Metabolites. To the dried metabolites (≤1 μg) in a 15-mL centrifuge tube or a Reacti-Vial (Pierce, Rockford, IL) was added 0.3 mL of dilute sulfuric acid in 2-propanol (0.5%); the tube was stoppered and allowed to stand at room temperature with magnetic stirring for 16 h. At the end of the reaction period, 0.5 mL of sodium phosphate (0.1 M, pH 7) was added and the solution was extracted twice with 1 mL of ethyl acetate. The combined ethyl acetate extracts were evaporated to dryness under nitrogen.

Fluorescence Derivatization of Metabolites. Dried metabolites were derivatized by reaction with 0.2 mL of a reagent solution consisting of acetic anhydride/1-methylimidazole/dimethylformamide (0.3:0.2:0.9 v/v/v) in a siliconized centrifuge tube (Sylon; Supelco, Bellefonte, PA) at 135 °C for 2 h as described previously for ivermectin and its polar metabolites (Tway et al., 1981; Chiu et al., 1986).

Reverse Isotope Dilution Assay (RIDA) of Unaltered [22,23-^{3}H]H_2B_{1a} and [22,23-^{3}H]H_2B_{1b} in Tissue Extracts. The unaltered drug was assayed by the HPLC–RIDA method developed in this laboratory (Chiu et al., 1985). The final tissue extract obtained from the above procedure was analyzed by RP-HPLC using solvent condition A to isolate the H_2B_{1a} and H_2B_{1b} components. The recovered column effluent corresponding to the drug peaks was evaporated to dryness and the remainder redissolved in ethanol for UV absorption measurements. Samples that required further purification were evaporated to dryness and rechromatographed either by RP-HPLC with solvent condition A or by NP-HPLC with solvent condition D.

High-Performance Liquid Chromatography. Liquid chromatographs from Spectra Physics (Model 8700) and Lab Data Control (Constametric I) were used in all the studies described in this paper. Each chromatograph was equipped with a sample valve (Rheodyne, Model 7120), a 100-μL sample loop, and a UV detector (Schoeffel Instruments, Model 770). A recorder–integrator (Hewlett-Packard, Model 3280) and a fraction collector (LKB, Model 2111) were connected to the SP 8700. The LDC unit was equipped with a fraction collector (LKB Ultrorac 7000) and a recorder (Linear Instruments). Fractions on HPLC charts were marked by an event-marking device installed between the detector and the fraction collector. All chromatographic separations were monitored by UV detection at 245 nm except for the fluorescence derivatives, which were monitored with a fluorometric detector (Schoeffel, Model FS 970) at an excitation wavelength of 364 nm and an emission wavelength of 440 nm. The flow rate was at 1 mL/min unless designated otherwise.

RP-HPLC Conditions. A Zorbax ODS analytical column (Du Pont, 4.6 mm × 25 cm) was used, with CO:Pell ODS guard column (Whatman). Chromatographies were carried out by isocratic or gradient elution with mobile phases consisting of acetonitrile/methanol/water. Composition (v/v/v) of the mobile phases and HPLC conditions were as follows: (A) 49.2:32.8:18; (B) 56.4:37.6:6.0; (C) 39:26:35 (0–10 min), 42:28:30 (10–22 min), 45:30:25 (22–35 min), 48:32:20 (35–48 min), 51:34:15 (48–70 min), 54:36:10 (70–100 min). Except with the SP 8700 HPLC ternary system, solvents were prepared by mixing water and premixed acetonitrile/methanol (6:4 v/v) at designated proportions.

NP-HPLC Conditions. Silica gel columns were used with an HC Pellosil packed guard column (Whatman). HPLC conditions were as follows: (D) Zorbax Sil analytical column (Du Pont, 4.6 mm × 25 cm), ethanol/isooctane (10:90 v/v), flow rate 1.0 mL/min; (E) Partisil M9 column (Whatman, 9.4 mm × 25 cm), ethanol/isooctane (15:85 v/v), flow rate 1.9 mL/min.

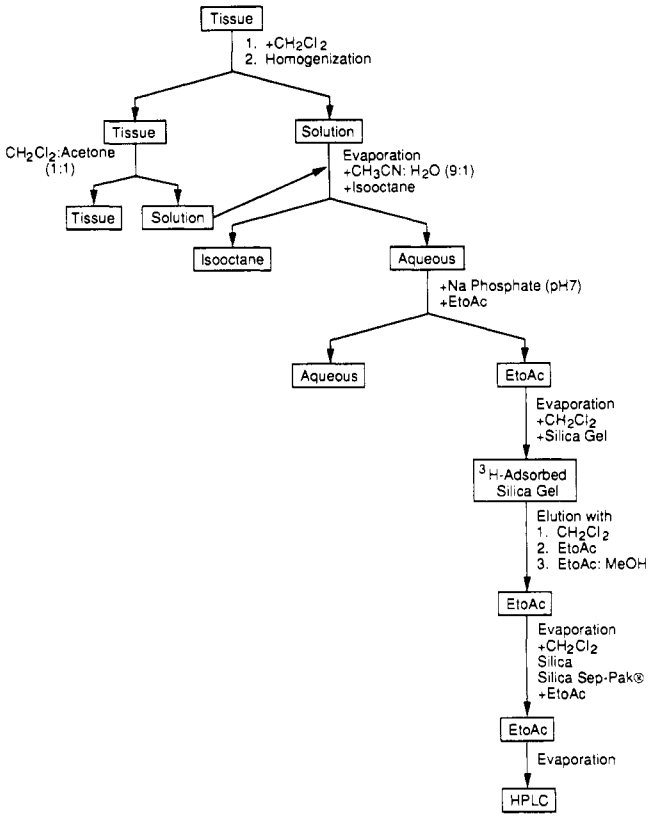

Figure 2. Procedure for the extraction of [22,23-^{3}H]ivermectin metabolites from 2 kg of liver from swine dosed subcutaneously at 0.4 mg/kg of body weight. Total radioactivity in the tissue calculated to 140 μg equivalents of ivermectin, of which ~45% was the parent drug.

Isolation of Major in Vivo Metabolites from 2 kg of Swine Liver. The procedure is summarized in Figure 2. Two groups of 7- and 14-day postdose liver samples were used for metabolite isolation. Livers from swine no. 9, 14, and 16 (7 days postdose) were 420, 411, and 280 g, respectively; those from swine no. 11 and 15 (14 day postdose) were 410 and 506 g, respectively. Livers from each group were composited separately by homogenization in a Waring blender with an equal volume of methylene chloride. The homogenates were extracted five times in two large separatory funnels with methylene chloride and finally with methylene chloride/acetone (1:1) until only background radioactivity was detected in the extract by counting aliquots. After filtration through fluted filter paper, the extracts were evaporated to dryness under reduced pressure. Each residue was redissolved in 5 × 50 mL acetonitrile/water (9:1) preequilibrated with isooctane and washed with equal volumes of isooctane each time. The acetonitrile extracts from the two groups were combined (~500 mL). About 750 mL of sodium phosphate buffer (0.1 M, pH 7) was added, and the solution was extracted with 3× equal volume of ethyl acetate. The combined extract was evaporated to dryness. The dry residue was redissolved in methylene chloride and passed through 10 g of silica gel as described above. The combined acetate washes after evaporation were repurified with three silica Sep-Pak cartridges connected in series and eluted with acetate and ethyl acetate/methanol (1:1). At each step of extraction, duplicate aliquots were removed for assay of radioactivity by scintillation counting.

RESULTS AND DISCUSSION

Radioactive Tissue Residue Distribution and Excretion. Tritium isotopic labels at $C_{22,23}$ positions have been shown to be stable under the biological conditions encountered in metabolism studies (Chiu et al., 1990); the distribution of [22,23-^{3}H]ivermectin-related radioactivity in swine tissues after a single subcutaneous dose of 0.4 mg/kg of body weight was studied in 12 animals, 3 each slaughtered at 1, 7, 14, and 28 days postdose. Table I shows the typical distribution of total radioactivity expressed as nanogram equivalents/gram of tissue (parts per billion) in 20 tissues and 2 fluids (bile and plasma). With the exception of the injection site muscle, the bile showed the highest radioactivity levels at all time points, indicating that biliary excretion was an important excretion route. The second highest levels were in fat and liver, with depletion half-lives of 5.2 and 5.1 days, respectively. Brain residue levels were the lowest at all times followed by those in plasma. By 28 days postdose, all tissue residue levels were <10 ppb, with many of them below the detection limit under the combustion conditions used in these experiments. These results are similar to those obtained from cattle, sheep, and rat (Jacob et al., 1983; Chiu et al., 1986, 1990).

Table I. Total Radioactive Residues (Drug Equivalents in Parts per Billion) in Tissues and Fluids from Swine Dosed Subcutaneously with [22,23-^{3}H]Ivermectin at 0.4 mg/kg of Body Weight[a]

	days postdose:	total residue, ppb			
		1	7	14	28
adrenals		19	23	6	1
bile		1342	1371	211	6
bone marrow		59	42	20	4
brain		3	3	1	0
cecum		32	35	9	1
colon		36	36	10	0
fat		265	169	45	6
heart		51	45	15	0
intestine (small)		80	39	10	1
kidney		69	72	18	1
liver		147	141	41	3
lung		60	56	17	0
lymph gland		39	33	12	2
muscle		33	30	9	0
pancreas		66	70	20	0
plasma		18	22	6	0
spleen		46	41	13	0
stomach		33	33	9	1
thymus		39	36	7	0
thyroid		47	23	14	2
tongue		32	24	11	1
injection site		18835	2928	4998	8

[a] One animal was analyzed at each time point. Animal numbers were 7 (1 day), 14 (7 days), 4 (14 days), and 17 (28 days). All values were averages of duplicate assays. Values below detection limits were reported as zero. Detection limits (ppb): plasma, 0.1; liver, 1.2, kidney, 0.8; muscle, 2.4–3.0; fat, 1.2.

As expected, the injection site samples possessed extremely high residue levels at 17–26 ppm 1 day after dosing (~1000-fold higher than plasma levels). Table II shows the depletion of injection site muscle radioactivity levels in 12 animals dosed subcutaneously with [22,23-^{3}H]ivermectin. Although the total radioactivity in the injection site samples (250 g, except for one animal) accounted for 46–79% of the dose at 1 day, its depletion was rapid. By 7 days postdose, the total residue was about 4.6% of the dose. By 28 days after withdrawal, only 0.02% of the dose remained at the injection site.

Recoveries of radioactivity by excretion were measured in urine and feces from three animals of the 28-day withdrawal group. Radioactivity excreted in urine was 0.4–0.6% of dose and in feces 29–30% of dose during the 7 days after dosing (Table III). These results were similar to those obtained from cattle, sheep, and rat; i.e., no more than 2% of the dose was excreted in urine, and the rest was recovered in the feces.

Unchanged Drug in Liver. The unchanged drug levels in edible tissues were determined by a reverse isotope dilution assay (RIDA) method developed in our laboratory

Table II. Total Radioactive Residue (Drug Equivalents in Milligrams) in Injection Site Samples from Swine Dosed Subcutaneously with [22,23-^{3}H]Ivermectin at 0.4 mg/kg of Body Weight[a]

barrow	withdrawal period, days	residue concn, ng/g	total residue in sample,[b] mg	dosed drug, mg	% dosed drug at injection site
7	1	18835	4.71	8.9	53.0
8	1	26093	6.52	8.3	79.0
12	1	16730	4.18	9.1	46.0
9	7	1628	0.405	9.3	4.4
14	7	2928	0.732	10.8	6.8
16	7	962	0.241	9.3	2.6
4	14	4998	1.24	9.9	12.5
11	14	139	0.035	9.4	0.37
15	14	28.7	0.0089	9.7	0.09
1	28	8.2	0.0021	8.7	0.02
2	28	5.6	0.0014	8.8	0.02
17	28	8.2	0.0021	8.8	0.02

[a] All injection site samples were repeatedly extracted with toluene before combustion assay, as described under Materials and Methods. [b] All injection site samples weighed 250 g except that from barrow 15, which weighed 310 g.

Table III. Excretion of Radioactivity in Feces and Urine of Swine Dosed Subcutaneously with [22,23-^{3}H]Ivermectin at 0.4 mg/kg of Body Weight Calculated as Percent of Dose[a]

		% dose recovered	
barrow	days postdose	feces	urine
1	1	0.58	0.03
	2	3.61	0.06
	3	9.24	0.10
	4	7.27	0.11
	5	5.08	0.09
	6	6.78	0.08
	7	6.56	0.09
	total	39.12	0.56
2	1	0.18	0.05
	2	1.39	0.03
	3	8.89	0.07
	4	5.16	0.05
	5	4.98	0.04
	6	13.70	0.04
	7	4.88	0.08
	total	39.18	0.36
17	1	1.38	0.04
	2	1.64	0.06
	3	3.30	0.07
	4	5.75	0.05
	5	5.01	0.04
	6	6.33	0.06
	7	5.39	0.05
	total	28.80	0.37

[a] Doses for barrows 1, 2, and 17 were 8.7, 8.8, and 8.8 mg, respectively.

previously (Chiu et al., 1985). For swine samples, the drug recovered by RP-HPLC of the tissue extract had to be rechromatographed on a NP column (silica) to obtain H_2B_{1a} and H_2B_{1b} of satisfactory purity on the basis of UV absorbance for specific activity determination. Table IV shows the percent of unchanged H_2B_{1a} and H_2B_{1b} in liver, fat, kidney, and muscle in 1-, 7-, and 14-days postdose animals. In all tissues, between 45 and 70% of the tissue residue was accounted for by the unchanged drug 1 or 7 days after dosing. It declined to ~30% in animals 14-days postdose. The depletion rate of the tissue residue calculated by fitting the residue levels by the least-squares method showed that the half-life of H_2B_{1a} was 3.8 days in both liver and fat and 5.4 days in kidney. Half-lives for H_2B_{1b} were 3.2, 4.0, and 4.6 days in liver, fat, and kidney, respectively. The half-life for radioactive residue in muscle was not calculated because drug levels were assayed at only two time points due to low residue levels. It is interesting to note that in swine the depletion half-lives of total residue and the parent drug were similar in liver and fat tissues. In comparison, a slower depletion rate was usually observed for fat residue in cattle, sheep, and rat (Chiu and Lu, 1989). This will be addressed in a later discussion.

Metabolites in Liver Tissue. In addition to the unchanged parent drug, radioactivity present in swine livers was due mainly to metabolites of slightly higher polarity than the parent, on the basis of elution sequences by RP-HPLC. As shown in Figure 3B, the major liver metabolites eluted in retention positions of 50–70 min under HPLC condition C. They were designated "druglike" metabolites to differentiate them from the polar metabolites 24-OH-H_2B_{1a} (V) and 24-OH-H_2B_{1b} (VI) and their respective monosaccharides (VII and VIII) found in the livers of cattle, sheep, and rat. At least five metabolites were present as resolved by HPLC and were designated D-1–5 sequentially with increasing polarity (Figure 3B). By normal-phase HPLC (condition D), metabolites eluted slower than the parent and were largely unresolved (Figure 3A). Because of the low concentrations of tissue residue, isolation and identification of the in vivo metabolites were difficult. Two approaches were taken to solve this problem: (1) isolate sufficient amounts of major metabolites for direct structural analysis if possible and (2) derivatize metabolites and compare with derivatives prepared from in vitro swine liver metabolites previously identified in our laboratories (Chiu et al., 1984). Two of these in vitro metabolites identified were 3″-*O*-desmethyl-H_2B_{1a} (IX) and 3″-*O*-desmethyl-H_2B_{1b} (X).

Concerning approach 1, the magnitude of the problem may be estimated as follows. On the basis of residue levels of 110 and 22 ppb from respectively 7- and 14-day postdose swine, and assuming that a metabolite accounting for 10% of total radioactive residue could be isolated with no loss, the amount of metabolite to be isolated from 1000 g of liver tissue from 7- and 14-day animals would be 11 and 2.2 μg, respectively. Direct structural analysis (eg., NMR, MS) may be carried out with these amounts only if sample purity is satisfactory. The actual isolation was carried out with 2 kg of the composite liver sample from 7- and 14-day postdose swine, containing about 2.4×10^7 dpm of tritium radioactivity in the initial, crude tissue extract. Of this amount of radioactivity, 88% (containing the parent and metabolites) was adsorbed by silica gel and subsequently was desorbed with solvents. The eluate containing essentially metabolites and some parent drug was 1.2×10^7 dpm (69 μg equivalents). This tissue extract sample was chromatographed first by NP-HPLC (condition E) to isolate the mixture of druglike metabolites as a group and then by repetitive two-dimensional HPLC using alternate RP-HPLC (condition A) and NP-HPLC (condition D) systems to resolve individual metabolites. Two major metabolites, D-2 and D-3, were isolated and purified, each at ~5 μg after HPLC chromatographies (Chiu et al., 1988b). Two-dimensional HPLC carried out as described not only enabled the purification of the major metabolites D-2 and D-3 but also established the HPLC capacity factors (k') of these metabolites under both NP- and RP-HPLC conditions. The k' values proved to be very useful in the identification of these metabolites.

For direct spectroscopic analysis, only limited information was obtained from D-2 and D-3 due to the low amount (~5 μg) available. Proton NMR of D-3 was weak,

Table IV. Total Radioactive Residues (Nanogram Equivalents/Gram of Tissue or Parts per Billion) and Percent of Unaltered Drug (H_2B_{1a} and H_2B_{1a}) in Edible Tissues of Swine Dosed Subcutaneously with [22,23-^{3}H]Ivermectin at 0.4 mg/kg of Body Weight[a]

		liver			fat			kidney			muscle		
days postdose	animal	total residue, ppb	H_2B_{1a}, %	H_2B_{1b}, %	total residue, ppb	H_2B_{1a}, %	H_2B_{1b}, %	total residue, ppb	H_2B_{1a}, %	H_2B_{1b}, %	total residue, ppb	H_2B_{1a}, %	H_2B_{1b}, %
1	7	147	36	19	265	38	13	69	32	13	33	50	22
	8	127	38	15	234	48	19	72	40	13	23	55	23
	12	322	ND[b]	ND	654	ND	ND	178	ND	ND	68	ND	ND
7	9	117	31	15	183	50	11	60	31	6	30	41	12
	14	141	31	24	169	50	14	72	32	11	30	37	9.2
	16	78	ND	ND	103	ND	ND	32	ND	ND	15	ND	ND
14	4	41	22	9	45	21	10	18	24	7	9	ND	ND
	11	15	18	4	21	28	9	7	ND	ND	2	ND	ND
	15	11	ND	ND	17	ND	ND	5	ND	ND	1	ND	ND
28	1	3	ND	ND	9	ND	ND	0	ND	ND	0	ND	ND
	2	2	ND	ND	4	ND	ND	0	ND	ND	0	ND	ND
	17	3	ND	ND	6	ND	ND	1	ND	ND	0	ND	ND

[a] Total radioactivity was assayed by combuation of duplicate tissue homogenate samples as described under Materials and Methods. All values represent averages of duplicates. Values below detection limits were reported as zero. See Table I for detection limits. [b] ND, not determined.

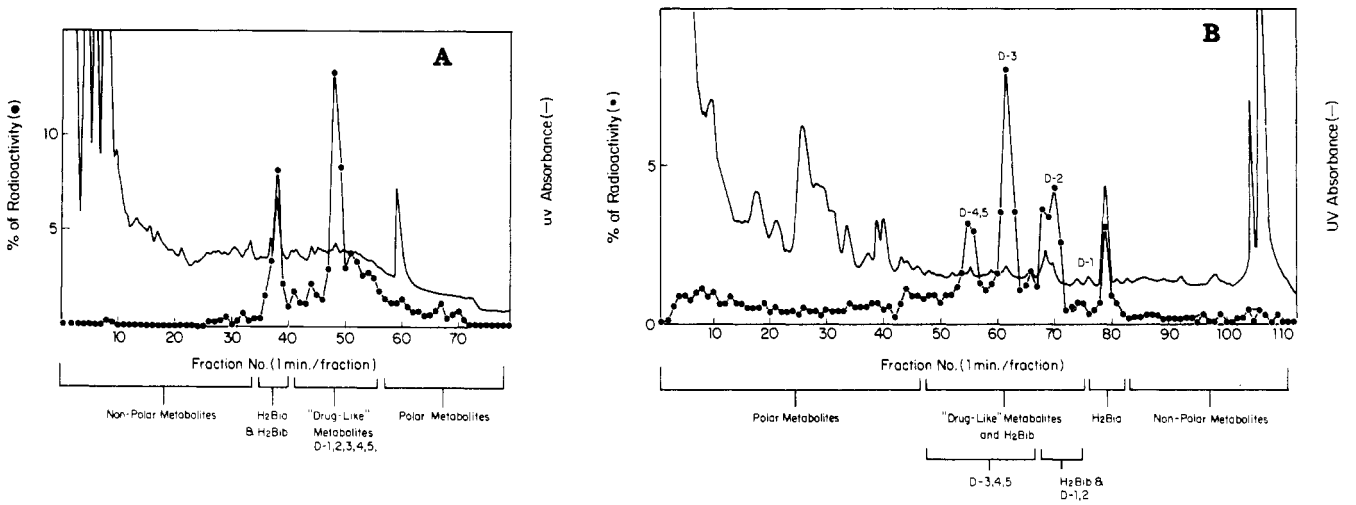

Figure 3. HPLC profile of [22,23-^{3}H]ivermectin residue in liver tissue extract of swine 14 days after dose, as analyzed with (A) normal-phase column, under HPLC condition D, and (B) reversed-phase column, under HPLC condition C.

and the only useful information one could derive from it was the presence of only one *O*-methyl group in the molecule as indicated by a singlet OCH_3 proton signal at 3.3 ppm. The position of this signal and the overall spectrum resembled the NMR spectra of the known monosaccharide of the drug (H_2B_{1a}-MS, III, and H_2B_{1b}-MS, IV) and the swine liver in vitro metabolites [3″-*O*-desmethyl-H_2B_{1a} (IX) and 3″-*O*-desmethyl-H_2B_{1b} (X)] (Chiu et al., 1984). The UV spectrum of D-3 was also measured. The spectrum was similar to those of ivermectin and the known metabolites IX and X, with λ_{max} at 245 nm. The UV absorption profile indicated that metabolite D-3 probably retained the same ring conjugation as the parent drug. FAB-MS was not too successful with D-3, probably due to the presence of fatty tissue contaminants in the sample. NMR and MS analyses were thus not attempted with metabolite D-2 because of the limited information derived from D-3. The chromatographic properties of D-2 and D-3, however, turned out to be extremely informative for structural elucidation. The capacity factors (k') of in vivo and in vitro metabolites from swine liver are listed in Table V. In the course of metabolite identification, the k' values of the in vitro metabolites served as a "library" of all potential biotransformation products. By comparison of the k' values of the in vivo and in vitro metabolites from RP-HPLC (first dimension), three compounds (a, c, and d) from the in vitro source were similar to the in vivo metabolite D-2 and analogously two compounds (g and h) to metabolite D-3. However, with additional k' values from NP-HPLC (second dimension), only one compound (i.e., c, or IX in Figure 1) from the in vitro metabolites chromatographed

Table V. Resolution of Ivermectin Metabolites by Two-Dimensional HPLC[a]

	k'		
	RP	NP	λ_{max}
H_2B_{1a} (I)	18	4	245
H_2B_{1a} (II)	12	4	245
H_2B_{1a}-MS (III)	9	6.3	245
H_2B_{1b}-MS (IV)	8	6.3	245
in vivo metabolites			
D-2	12	10	
D-3	9	10	245
in vitro metabolites from			
H_2B_{1a}-a	12	6.3	245
H_2B_{1a}-b		8	
H_2B_{1a}-c (IX)	12	10	245
H_2B_{1a}-d	12	5	280
H_2B_{1a}-e	11	6.7	245
H_2B_{1b}-f	12	5	245
H_2B_{1b}-g	9	7	245
H_2B_{1b}-h (X)	9	10	245

[a] HPLC conditions were RP, condition A, and NP, condition D.

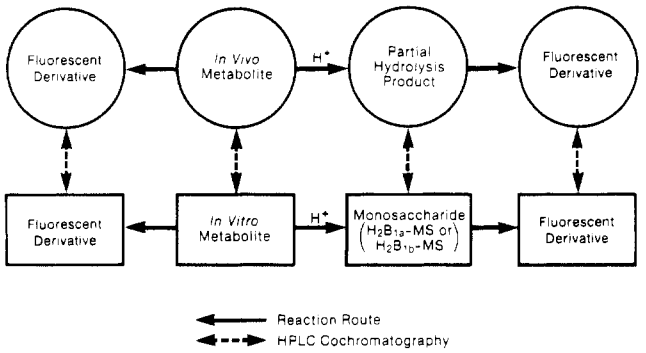

Figure 4. Routes of identification of swine liver metabolites. Solid arrows designate derivatization reaction routes; dotted arrows designate comparisons by mixed-sample HPLC cochromatography.

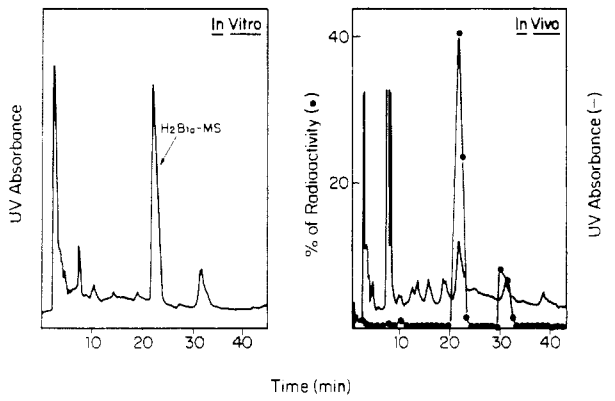

Figure 5. HPLC profile of swine liver in vitro metabolite 3″-*O*-desmethyl-H_2B_{1a} (IX) and in vivo metabolite D-2 after partial acid hydrolysis. Note the identical retention time of the hydrolyzed products from IX (UV peak) and D-2 (radioactivity peak).

identically with the in vivo metabolite D-2. Analogously, only one in vitro metabolite (h, or X in Figure 1) possessed k' values identical with those of metabolite D-3 in both dimensions. These results strongly suggested the identity of D-2 as IX and D-3 as X, thus ruling out the possibility of the compounds being monosaccharides. The latter structures would have been assigned if comparisons were made on the basis of RP-HPLC or NMR data alone. Confirmation of the identity was achieved by first cochromatography of metabolite D-2 and D-3 with IX and X, respectively, followed by two separate chemical derivatization reactions (Figure 4). Metabolite D-3 was converted to a fluorescent derivative by a base-catalyzed derivatization reaction in the presence of acetic anhydride. This derivative was identical chromatographically with the derivative prepared from 3″-*O*-desmethyl-H_2B_{1b} when analyzed with HPLC condition B. Independently, D-3 was also subjected to partial acid hydrolysis in dilute sulfuric acid. The hydrolysis product upon RP-HPLC analysis (condition A) was shown to be identical with the hydrolysis product of 3″-*O*-desmethyl-H_2B_{1b}, i.e., H_2B_{1b}-MS (IV). Similar results were obtained with metabolite D-2 and in vitro metabolite 3″-*O*-desmethyl-H_2B_{1a} [which produced H_2B_{1a}-MS (III) upon hydrolysis]. The HPLC comparison of hydrolysates of D-2 and metabolite IX is shown in Figure 5.

Further comparison was carried out with fluorescent derivatives of the acid hydrolysate of D-2 or D-3, which again proved to be identical chromatographically (HPLC condition B) with derivatives of its respective in vitro metabolite IX or X. Results of these studies firmly established the identities of the in vivo and in vitro metabolites. On the basis of the radioactivity profiles and the isolation recovery, each of these major metabolites was estimated to account for ~12% of total residue in the composite liver tissue. The distribution of liver residue is summarized in Table VI. Due to the small quantities isolated, structures of other metabolites D-1, D-4, and D-5 have not been elucidated to date. As shown in Table VI, the group of druglike metabolites accounted for 38% of total radioactivity in the 7- and 14-day postdose composite sample, compared to 45% of the parent drug.

Table VI. Classification of Total Radioactive Residues in Composite Liver Tissue of Swine Based on Extraction and HPLC Fractionation[a]

radioactive group	residue polarity	isolation fraction	% total radioactive residue	
I	polar	EtOAc/MeOH eluate of silica gel	2	5
II	polar	MeOH eluate of Sep-Pak	3	
III	druglike metabolites			
	D-5	HPLC	2	38
	D-4	HPLC	8	
	D-3[b]	HPLC	12	
	D-2[b]	HPLC	12	
	D-1	HPLC	4	
IV	unchanged drug	(1) methylene chloride eluate of Sep-Pak	15	45
		(2) HPLC	30	
V	nonpolar	(1) methylene chloride eluate of silica gel	10	12
		(2) HPLC	2	
			total	100

[a] Swine slaughtered 7 and 14 days after subcutaneous dosing at 0.4 mg/kg of body weight. [b] Structures identified: D-2 as 3″-*O*-desmethyl-H_2B_{1a} (IX); D-3 as 3″-*O*-desmethyl-H_2B_{1b} (X).

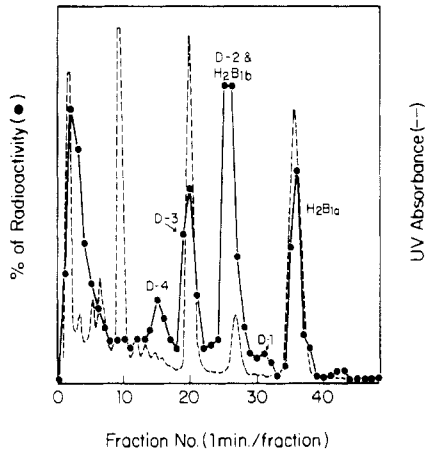

Figure 6. Reversed-phase HPLC profile of [22,23-^{3}H]-ivermectin residue in swine fat tissue. The tissue extract was from 14-day postdose animals, chromatographed with added unlabeled H_2B_{1a} (I), H_2B_{1b} (II), and H_2B_{1a}-MS (III). HPLC condition A was used.

Metabolites in Swine Fat. In contrast to cattle, sheep, and rat, swine do not follow the metabolism pattern observed in the fat tissue of other species, i.e., formation of fatty acid conjugates of hydroxylated ivermectin (Chiu et al., 1988a). The fat metabolite profile of swine resembled that of the liver tissue; i.e., two major metabolites slightly more polar than the parent drug were present (Figure 6). These were confirmed as the 3″-*O*-desmethyl derivatives of H_2B_{1a} and H_2B_{1b} by chromatographic comparison with authentic samples. The absence of ester conjugation of swine liver metabolite in the fat tissue can probably be attributed to the absence of a primary hydroxyl functional group in these molecules. As a result, these compounds

are probably less favorable substrates for esterification compared to 24-OH-H_2B_{1a} and -H_2B_{1b} produced in the livers of cattle, sheep, and rat. The similarity of liver and fat metabolite profiles in swine also explains the close resemblance of the residue depletion half-lives (5 days) in these two tissues of swine. In other species, formation of nonpolar conjugates apparently lengthened the depletion time of radioactive residues in the fat relative to that in the liver.

In summary, we have demonstrated that when [22,23-^{3}H]ivermectin was dosed subcutaneously in swine at 0.4 mg/kg of body weight, essentially the same excretion and tissue residue distribution pattern as those found in cattle, sheep, and rats were observed. The metabolism of the drug, however, was different in swine. In both liver and fat tissues, the major metabolites were *O*-demethylation derivatives of ivermectin, although the parent compounds were still the major radioactive residue at early time points, e.g., within 7 days after dosing. Good correlation has been shown between in vitro and in vivo metabolism of ivermectin in swine, as has been concluded from other animal species in our earlier papers.

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